Biochemical, cell biological and immunological issues surrounding the endoplasmic reticulum chaperone GRP94/gp96

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The past year has borne witness to compelling demonstrations of the utility of peptide complexes with glucose regulated protein 94 (GRP94, also known as gp96) in cancer immunotherapy. Insights into the structural basis of peptide binding to GRP94 have been obtained and the role of the transporter for antigen presentation in defining the GRP94-bound peptide composition has been determined.

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Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>BiP</td>
<td>binding protein</td>
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<td>BMDC</td>
<td>bone marrow-derived dendritic cells</td>
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<td>CTL</td>
<td>cytotoxic T cell</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>GRP94</td>
<td>glucose regulated protein 94</td>
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<td>hsp90</td>
<td>heat shock protein 90</td>
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<td>PDI</td>
<td>protein disulfide isomerase</td>
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<td>T2</td>
<td>TAP-deficient cell lines</td>
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<td>TAP</td>
<td>transporter associated with antigen presentation</td>
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<td>VSV</td>
<td>vesicular stomatitis virus</td>
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Introduction

The endoplasmic reticulum (ER) paralog of the hsp90 (heat shock protein 90) family of chaperones, glucose regulated protein 94 (GRP94) (also known as gp96), has recently garnered considerable attention for its potent, and perhaps unexpected, ability to elicit CD8+ cytotoxic T-cell responses to its bound peptide pool [1-4]. Of particular interest, cancer cell derived GRP94 has recently been shown to stimulate strong autologous, anti-tumor immune responses to pre-existing cancers [5-11]. It is known that GRP94 antigenicity derives solely from the bound-peptide fraction [2,3,6]. Furthermore, the broad, yet autologous, anti-tumor activity of cancer-derived GRP94 indicates that GRP94 associates with a population of peptides that is both representative of a given cell type and, at least in part, suitable for expression on MHC class I molecules [5-6,7-8]. These observations have significant bearing on future research into the therapeutic utility of chaperone–peptide complexes in cancer and viral immunotherapy as well as the cell biology of antigen processing and peptide traffic in the ER.

The structure–function relationships that identify GRP94 as a peptide-binding protein are unknown. Recent crystal structure studies of cytosolic hsp90 do, however, provide for interesting speculation on the structural organization of GRP94 [9,10,11]. This review will examine the past year’s advances in the structural basis of GRP94 function, its ever-expanding application in cancer immunotherapy, and the cell biological significance of the CD8+ T cell priming that accompanies immunization with GRP94–peptide complexes.

The phylogenetic and subcellular distribution of GRP94

Hsp90 proteins are ubiquitously expressed in the phyla Eucarya and can occur in both cytosolic and endoplasmic reticulum-resident forms. Phylogenetic analyses support a model in which GRP94, the ER paralog of hsp90, arose through an ancient gene duplication event (Figure 1) [12]; however, within the Eucarya, GRP94 has only been identified in the Metazoa. Extensive homology searches of the Saccharomyces cerevisiae genome, for example, indicate that S. cerevisiae does not contain a GRP94 homolog (C. Nicchitta, P. Wearsch, unpublished data). That GRP94 has not yet been identified in a unicellular eukaryote suggests that the cellular function(s) performed by GRP94 are perhaps unique to multicellular eukaryotes. The validity of this conclusion awaits genome analysis of primitive unicellular eukaryotes, such as Giardia, but is suggestive of a divergence in ER function that accompanied the appearance of multicellular, differentiated organisms.

As is characteristic of proteins resident to the lumen of the ER, GRP94 is synthesized with an amino-terminal signal sequence and a carboxy-terminal ER retention/retrieval signal [13-16]. When ER membranes are mechanically disrupted, permeabilized with detergents or exposed to alkali buffers, GRP94 is released in parallel with other lumenal proteins, such as the ER hsp70 homolog (BiP), protein disulfide isomerase (PDI), calreticulin and the 72 kDa ER protein ERp72, indicating that it functions as a soluble, luminal component of the ER [13,17,18].

Despite the existence of canonical ER localization signals, a partial, albeit minor, fraction of GRP94 has been observed to localize to the cell surface ([19]; Y. Argon, personal communication). The mechanistic basis for this altered localization and its functional consequence(s) remain to be determined. It should be noted that such altered localization has been seen with transformed cells, but not as yet in nontransformed cells.
Phylogenetic analysis of Hsp90 proteins. The relative sequence divergence between members of the hsp90 protein family were analyzed by the CLUSTAL method, using the PAM250 weight table, as provided on the DNASTAR Lasergene software package (DNASTAR, Madison, WI, USA). The ordinate scale represents per cent sequence divergence. The accession numbers of the sequences analyzed were as follows: hamster hsp90α, L33676 (GenBank); human hsp90α, P07900 (SwissProt); rat hsp90β, S45392 (GenBank); human hsp90β, P08238 (SwissProt); D. discoideum, hsc90, L43591 (GenBank); S. cerevisiae, hsc82, P15108 (SwissProt); S. cerevisiae, hsp82, P02829 (SwissProt); canine GRP94, U01153 (GenBank); murine ERp99, P08113 (SwissProt); Caenorhabditis elegans, GRP94, Z69751 (GenBank); barley GRP94, S31962 (GenBank); and E. coli, HtpG, P10413 (SwissProt).

**GRP94 as a peptide-binding protein: a tale of tumor antigens**

Numerous experimental observations have indicated that GRP94 displays intrinsic (poly)peptide-binding activity. GRP94 was first identified as a gene product whose synthesis is upregulated upon glucose starvation and in response to various experimental treatments that induce the accumulation of unfolded proteins in the ER [20–22]. Consistent with its proposed chaperone activity, GRP94 has been identified in association with folding intermediates of a number of oligomeric proteins [23–26]. The precise role of GRP94 in protein folding in the ER remains, however, to be elucidated. In this regard, it will be of particular interest to identify the structural determinants recognized by GRP94. Recent data suggest that GRP94 may preferentially recognize late stages of protein folding intermediates [27].

In 1989, in studies of the ER hsp70 protein BiP, Flynn et al. [28] demonstrated that short peptides can be used to mimic discrete protein segments and that BiP–peptide interactions displayed characteristics identical to those seen with protein folding intermediates. The possibility that ER chaperones may actually function, at least in part, as peptide-binding proteins was also suggested by the observation that PDI displays a broad specificity peptide-binding activity [29]; however, the first indication that the peptide-binding properties of ER chaperones and protein-folding enzymes may be of biological relevance was provided by a remarkable series of observations from the Srivastava laboratory. Srivastava and co-workers [30] were the first to demonstrate that GRP94 isolated from a chemically induced tumor and injected into syngeneic mice produced protective immunity to subsequent challenges with cells from the parent tumor. The tumor rejection activity of GRP94 is critically dependent upon the bound peptide fraction and was demonstrated to reflect a CD8+ cytotoxic T cell (CTL) response, which is of high immunological and cell biological interest [1].

Recent reports have convincingly established the utility of GRP94–peptide complexes in eliciting cellular immune responses. In addition to the clear activity of MethA sarcoma-derived GRP94 in eliciting protective immunity, GRP94 has been demonstrated to induce specific CTL responses to intracellular antigens [2], minor H-antigens [2] and viral antigens [31]. The specificity of such responses was convincingly illustrated in studies in which isolated macrophages, pulsed with GRP94 and restituted in vitro with a known peptide epitope of the vesicular stomatitis virus (VSV)8, were recognized by VSV-specific CTLs [3]. This observation has been elaborated in a recent study demonstrating the epitope-specific antigenicity of GRP94 loaded in vitro with peptides of differing primary sequence and origin [6**]. Consistent with previous studies, CD8+ T cells from mice immunized with the defined GRP94–peptide complexes displayed epitope-specific CTL activity [6**].

The capacity of GRP94 to bind a diverse array of peptides has prompted investigations into the possibility that the GRP94-bound peptide fraction may be representative of the panoply of peptides that are trafficked into, or generated within, the ER. This question has been investigated in three recent studies. Lammert et al. [7] used photoreactive peptide derivatives to investigate the requirement for the transporter associated with antigen presentation (TAP) for peptide binding to GRP94. Using peptides known to require TAP for transport in the ER and either TAP-deficient cell lines (T2) or T2 cells transfected with rat TAP1 and TAP2, Lammert et al. demonstrated that GRP94 can associate with peptides whose transport into the ER is TAP dependent [7]. In a similar experimental approach, Spee and Neefjes [32**] identified GRP94, PDI, calreticulin, and two as yet unidentified proteins (gp120, gp170) as peptide-binding proteins of the ER lumen. Furthermore, through the use of a family of nonamer peptides, in which the residues at the 2 and 9 positions were systematically altered, Spee and Neefjes demonstrated that GRP94 displayed a distinct bias for peptides containing uncharged amino acids at the anchor residue positions [32**]. In addition, studies in which GRP94 was isolated from β-galactosidase overexpressing cells with and without TAP2 and screened in CTL assays for the induction of CTL responses, indicated that GRP94 can associate with peptides whose
transport into the ER is TAP dependent (β-gal), and TAP2 independent (a subclass of minor H antigens) [8**].

Until recently, GRP94–peptide complexes have been investigated solely with regard to the induction of protective immunity. A number of groups are now investigating the utility of GRP94–peptide complexes in the immunotherapy of pre-existing cancers. Tamura et al. [5**] have demonstrated that immunization of tumor-bearing mice with tumor-derived GRP94 can dramatically slow metastatic progression [5**]. Significantly, GRP94 derived from metastatic lesions, which may differ in various histological criteria from the parent tumor, are also highly effective in eliciting an anti-tumor immune response [5**]. In this study, tumor-derived GRP94–peptide complexes elicited a substantial reduction in tumor burden in tumors of differing histological origin, modality of induction, haplotype and inherent immunogenicity. These data provide a compelling argument for the efficacy of heat shock protein–peptide complexes in the induction of cell–specific immune responses.

With the recent heightened interest and appreciation of the role of professional antigen-presenting cells (APCs) in the regulation of cellular immune responses [33,34], a number of groups are evaluating GRP94-pulsed, bone marrow-derived dendritic cells (BMDC) as immunotherapeutic vaccines [35,36]. In this approach, BMDC are pulsed with tumor-derived GRP94–peptide complexes and the transfer of the GRP94-derived peptides to BMDC MHC class I molecules assayed through CTL assays and in tumor regression models. We have evaluated the efficacy of GRP94-pulsed BMDC in a melanoma model for pre-existing cancer. In these experiments, mice were injected with cells from the poorly immunogenic, highly metastatic melanoma clone, B16/F10.9. Following the development of a palpable tumor (~5.5–7.5 mm), the tumor was surgically resected and the mice were immunized with phosphate-buffered saline, irradiated B16/F10.9 cells, or BMDC pulsed with B16/F10.9-derived GRP94. As depicted in Figure 2, tumor removal alone does not block metastatic progression. Following sacrifice at 30 days, mice display a substantial tumor burden, as illustrated by the fivefold increase in mean lung weight (Figure 2). Furthermore, immunization with irradiated B16/F10.9 cells was without therapeutic benefit, as has been previously reported [37]. In marked contrast, mice immunized with F10.9 GRP94-pulsed BMDC display mean lung weights nearly identical to those of normal mice (Figure 2). These data demonstrate that immunization with tumor-derived GRP94-pulsed BMDC can dramatically inhibit metastatic tumor progression and suggest procedures by which professional APCs can be used in cancer immunotherapy.

**The regulation of GRP94 peptide-binding activity**

The results of immunization studies, as well as in vitro investigations into GRP94 peptide-binding activity, indicate that GRP94 can associate with a diverse array of peptide substrates [2,6**,7**,8**,32**,38**,39**]. At present, the sequence and size determinants for peptide binding are unknown; these questions are, however, under active investigation in a number of laboratories. Similarly, little is currently known about the regulation of GRP94 peptide-binding activity. In this regard, it should be noted that GRP94–peptide complexes appear to be very long-lived. Thus, the bound peptide fraction, or at least a subset thereof, remains in stable association with GRP94 throughout conventional protein purification procedures. Furthermore, it has recently been demonstrated that, once bound, the peptide–GRP94 complex is SDS-resistant [6**]. That said, it is evident that following uptake by professional APCs, GRP94-bound peptides are exchanged onto MHC Class I molecules. It follows, therefore, that peptide exchange from GRP94 to MHC class I molecules is possibly regulated, or activated, in a compartment-specific manner.
In addition to the vagaries surrounding the peptide-binding specificity of GRP94, a defined peptide-binding pocket has not been identified. Recent crystal structure studies on the amino-terminal domain of hsp90 have provided some interesting, albeit inconsistent, insights into potential ligand binding domains of GRP94. Stebbins et al. [11**] reported the crystal structure of the amino-terminal domain of human hsp90 and noted an α+β sandwich structure that encompasses a highly conserved region of the family of hsp90 proteins. On the basis of molecular modeling, Stebbins et al. [11**] postulated that this domain forms a binding pocket suitable for a five amino acid polypeptide in a turn conformation. Crystal structure studies of the identical domain from yeast Hsp82, the yeast Hsp90 homolog, have yielded an alternative interpretation of its structure and function [9*,10**]. The crystal structure of the yeast amino-terminal domain was identified as a domain-swapped dimer, with a central cavity residing between the two subunits forming a binding pocket or ‘molecular clamp’ [9*]. In a subsequent crystal structure study, the amino-terminal dimer domain was crystallized in complex with either ADP or ATP and it was observed that the conserved α+β sandwich region served as an ATP/ADP binding site [10**]. Prodromou et al. have also noted that the central binding pocket is of appropriate dimensions for the binding of nonameric peptides in an extended conformation [10**]. The results of Prodromou et al. [10**], detailing an ATP/ADP binding site for the hsp90 amino-terminal dimer, raise a number of rather perplexing questions regarding the ribonucleotide-binding properties of the hsp90 proteins [10**]. To wit, do the hsp90s, and in particular GRP94, bind and hydrolyze ATP and does ATP binding and hydrolysis regulate the interaction of GRP94 with (poly)peptide substrates? When investigated through use of photolabeling, GRP94 was identified as an ATP-binding protein [40,41]. Direct kinetic analyses of ATP binding to GRP94 indicated, however, that binding was vastly substoichiometric, and could be ascribed to a high specific activity, low enrichment (<0.1%) ATPase contaminant of pharmacological similarity to casein kinase II [37]. Furthermore, neither ATP nor ADP have an effect on the peptide-binding activity of GRP94 when assayed at substrate saturation, or under pseudo first-order reaction conditions [38*], (PA Wearsch, CN Nicchitta, unpublished data). In addition, it has proven exceedingly difficult to identify the specific binding of ATP to both cytosolic hsp90 and GRP94. Using spin-labeled ATP analogs, for example, Scheibel et al. [42*] observed half maximal binding of ATP to human hsp90 at 400µM ATP. It has not yet been determined whether GRP94 displays identical, very low affinity ATP binding. At present, the preponderance of experimental evidence indicates that neither ATP nor ADP serve an essential function in the regulation of GRP94 activity.

Analysis of GRP94 structure by hydrodynamic and electron microscopic approaches indicated that the molecule is a tri-nodular rod comprised of two subunits oriented in a direct, antiparallel array (Figure 3) [43]. The orientation is conferred by a carboxy-terminal oligomerization domain that maintains the amino termini at opposing ends of the molecule, a conformation seemingly unlikely to form intramolecular amino-terminal dimers (Figure 3) [43].

**Cell biological and immunological implications**

The capacity of GRP94–peptide complexes to elicit a cellular immune response is dependent upon the transfer of the GRP94 associated peptides to MHC class I molecules of professional APCs [1–3,8**]. As this requires that extracellular GRP94 gain access to the class I antigen processing pathway, the observation that GRP94–peptide complexes can elicit a class I response becomes of fascinating cell biological and immunological interest, particularly with respect to the phenomenon of cross-priming. The ability of professional APCs to mediate the transfer of extracellular antigens to the class I pathway provides a probable explanation for how class I-associated antigens are presented to naïve T cells in the lymphoid organs, but begs the question of pathway. These data notwithstanding, the precise localization of the peptide binding site(s) on GRP94 remains to be defined.

To obviate inappropriate tissue destruction, antigen processing into the class I and class II pathways is compartmentally segregated, such that extracellular antigens are processed for expression on class II molecules and intracellular antigens on class I molecules. Given the central importance of professional APCs in the
activation of naive CD8+ T cells, it is apparent that there exists a mechanism(s) by which peptides associated with GRP94 can access the class I antigen processing pathway. Indeed, there are numerous reports of the class I presentation of exogenous antigens, primarily involving antigens presented in particulate form [44-46]. As demonstrated by Suto and Srivastava [3], however, GRP94-associated peptides are presented on class I molecules upon pulsing of macrophages with a fully soluble (100,000 x g supernatant) preparation of GRP94. How, then, is GRP94 internalized by professional APCs?

Day et al. [47**] have identified a novel pathway for the direct delivery of class I-suitable peptides from the extracellular space to the lumen of the ER. Although this pathway is unable to mediate the trafficking of a soluble protein, such as β2-microglobulin, to the ER, Day et al. [47**] do note that the vesicular trafficking intermediates arise from the plasma membrane [46]. Should such plasma membrane derived vesicles contain membrane receptors for GRP94, a direct transfer of GRP94–peptide complexes from the extracellular space to the ER could occur. That dendritic cells display a mannose receptor, and recognizing that GRP94 is a high-mannose glycoprotein, suggests one likely pathway for the receptor-mediated entry of GRP94 into professional APCs. There exist other, and likely overlapping, trafficking alternatives. Norbury et al. [48**,49] have provided compelling evidence that bone marrow-derived macrophages and dendritic cells can constitutive macropinocytosis. With respect to GRP94, and as mentioned previously, uptake through constitutive macropinocytosis would require the release (regulated?) of GRP94-bound peptides in the cytosol for subsequent entry into the ER.

Perhaps internalized GRP94 is trafficked within the secretory pathway to the ER. It is known, for example, that (poly)peptides bearing a carboxy-terminal KDEL motif, once they have gained access to the trans-Golgi network, may associate with Erp2, the KDEL receptor, and undergo retrograde trafficking to the ER [50]. It may also be considered that, following internalization, either through receptor-mediated or bulk phase pathways, GRP94–peptide complexes would be expected to access the endosomal network wherein, because of the acidic environment, peptide release could be stimulated. The transport of endosomal-free peptides to the cytosol would, however, require an as yet unidentified mechanism for transendosomal peptide transport. The formal possibility that peptide exchange between GRP94–peptide complexes and empty, cell-surface class I molecules is responsible for class I expression also deserves mention.

Conclusions
A number of recent publications have provided compelling evidence for the utility of GRP94–peptide complexes in cancer immunotherapy. Although it had been established that immunization with GRP94 can elicit protective immunity, recent studies have now demonstrated that GRP94-based immunotherapy is effective in models of pre-existing cancer [5**]. The potential application of GRP94–peptide complexes in immunotherapy is further heightened by results indicating that GRP94 can associate with peptides whose appearance in the lumen of the endoplasmic reticulum is governed by TAP-dependent and TAP-independent processes [7*,8**,51]. On the basis of these findings, it is apparent that the GRP94-associated peptide pool is representative of the entire population of ER-associated peptides. Studies on the mechanism and regulation of peptide binding by GRP94 are currently underway and, in combination with recent crystal structure studies of the structural domains of the hsp90 proteins, are expected to provide a molecular description of the structural and regulatory basis for peptide binding. At present, the mechanism of peptide exchange of GRP94-associated peptides onto MHC class I molecules is unknown, and could represent a novel trafficking pathway that is perhaps unique to professional APCs. The coming years of research into the mechanism of GRP94 action are likely to provide exciting new insights into the regulation of antigen processing in professional APCs, peptide traffic in the ER, and the molecular basis of hsp90 protein function.

Acknowledgements
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References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest


A compelling study demonstrating that vaccination with tumor-derived heat shock protein–peptide complexes elicits a CD8+ and CD4+ response against the parent tumor, as well as metastatic lesions. Tumor-derived heat shock protein–peptide complexes are shown to be effective immunotherapeutic agents against spontaneous, chemically induced and UV light-induced cancers. This constitutes the first demonstration that heat shock protein vaccines are effective in models of pre-existing cancer.

Glucose regulated protein (GRP94) and gp96 are reconstituted in vitro with various synthetic peptides and shown to elicit peptide-specific CD8+ responses. Data are presented demonstrating prophyllactic immunization against a lymphoma variant expressing the nucleocapsid protein of vesicular stomatitis virus (VSV), following vaccination with GRP94 reconstituted in vitro with the dominant immunogenic epitope of VSV.


Using paired cell systems differing in the transporter associated with antigen transport (TAP) expression, the authors demonstrate that endoplasmic reticulum (ER)-localized GRP94/gp96 binds peptides trafficked to the ER in a TAP-dependent manner. This report clearly demonstrates that GRP94/gp96 participates in peptide trafficking in the ER and raises interesting questions concerning the fate of peptides that are transported by TAP, yet escape assembly onto class I molecules.


In investigating the transporter associated with antigen presentation (TAP) dependence for peptide loading onto GRP94/gp96, the authors have discovered that GRP94/gp96 can associate with a diverse array of peptides regardless of whether the peptides are transported in a TAP-dependent or TAP-independent manner. These data demonstrate that the GRP94/gp96-bound peptide fraction is wholly independent of the MHC haplotype and suggest that this fraction is broadly representative of the endoplasmic reticulum–associated peptide pool.


The crystal structure of a recombinant amino-terminal domain of yeast hsp90 reveals a domain-swapping dimer that includes a potential "molecular clamp" region lying within the intrasubunit groove. These results suggest both a potential site of hsp90–substrate interaction and a structural mechanism for regulating substrate binding and release.


When co-crystallized with ATP or ADP, a highly conserved region of the amino-terminal domain of yeast hsp90 reveals a domain-swapping dimer that includes a potential "molecular clamp" region lying within the intrasubunit groove. These results suggest both a potential site of hsp90–substrate interaction and a structural mechanism for regulating substrate binding and release. For both nucleotides, the measured affinities are quite low, perhaps suggesting a structural, rather than enzymatic, contribution to hsp90 function.


The co-crystal structure of the anti-tumor agent geldanamycin and the amino-terminal domain of hsp90 reveals a monomeric quaternary structure and a hydrophobic binding pocket within a highly conserved region of the hsp90 family of proteins. The geldanamycin binding site is suggested to mimic a binding site for a pentameric peptide domain and may therefore describe the peptide binding site of the native protein.


23. Melnick J, Avet S, Argon Y: The endoplasmic reticulum protein GRP94, in addition to BiP, associates with ubiquitin-protein conjugates, regardless of whether the peptides are transported in a TAP-dependent or TAP-independent manner. These data demonstrate that the GRP94/gp96-bound peptide fraction is wholly independent of the MHC haplotype and suggest that this fraction is broadly representative of the endoplasmic reticulum–associated peptide pool.


Photoaffinity peptide analogs were used to identify gp96, protein disulfide isomerase and calreticulin as peptide-binding proteins of the endoplasmic reticulum lumen. Of particular note, screening of a peptide library containing degenerate residues at the 2 and 9 positions indicated a distinct bias by gp96 for peptides containing uncharged amino acids at these positions. These data are the first description of a peptide binding specificity for gp96.


51. Marusina K, Reid G, Gabathuler R, Jeffries W, Monaco J J: Novel peptide-binding proteins and peptide transport in normal and TAP-deficient microsomes. Biochemistry 1997, 36:858-863. Using photo-labeled peptide analogs, a number of endoplasmic reticulum (ER)-specific peptide binding proteins are identified including glucose regulated protein (GRP-94, p96, and a complex of lower molecular weight proteins specific for N-linked sugar bearing peptides (p36 complex). This is an important study that will contribute to the elucidation of the regulation of peptide transport into, and out of, the ER lumen. In addition, data are presented demonstrating sequence-specific peptide transport in the presence of transporter associated with antigen processing (TAP)-1 alone.